EXPRESSION PATTERN OF DRUG-RESISTANCE GENES IN MATCHED CLINICAL ISOLATES OF *CANDIDA ALBICANS* AT VARIABLE FLUCONAZOLE EXPOSURE TIMES

A THESIS IN

Cellular and Molecular Biology

Presented to the Faculty of the University
of Missouri-Kansas City in partial fulfillment of
the requirements for the degree

MASTER OF SCIENCE

by

ERIC STEPHEN GEANES

B.S., Kansas State University, 2014

Kansas City, Missouri

2016

EXPRESSION PATTERN OF DRUG-RESISTANCE GENES IN MATCHED CLINICAL ISOLATES OF *CANDIDA ALBICANS* AT VARIABLE FLUCONAZOLE EXPOSURE TIMES

Eric Stephen Geanes, Candidate for Master of Science

University of Missouri – Kansas City, 2016

ABSTRACT

Candida albicans is an opportunistic fungus that exists naturally in the vaginal, urinary, and gastrointestinal tracts of humans. Typically, this yeast remains balanced with the body's other natural microbial flora and the host immune system. However, infections may occur in individuals with compromised immune systems. A common drug used to treat these infections is fluconazole (FLC), a fungistatic azole targeting lanosterol 14α-demethylase (Erg11) in the ergosterol biosynthesis pathway. In some clinical isolates, resistance to FLC is due to overexpression of a) the encoding genes *CDR1* and *CDR2* for the ABC Transporters, b) the Major Facilitator Transporter encoding gene *MDR1*, or c) *ERG11,* the azole target gene. Previous studies have shown gene expression in a matched set of clinical isolates is maximized at the drug concentration that matches the minimum inhibitory concentration (MIC_{80}) of the strain to FLC (1).

This study investigates the gene expression levels of the drug resistant genes in selected *C. albicans* isolates from the matched series in the presence of their corresponding MIC_{80} to FLC at different times in the growth cycle. The four isolates used in this study were selected from a series of 17 isolates all taken from the same patient. Quantitative real time

PCR (qRT-PCR) was used to measure mRNA expression of the aforementioned genes in the presence and absence of FLC at their respective MIC_{80} concentrations at growth times ranging from 1 hour to 32 hours. Gene expression in the presence of drug was compared to the respective expression levels in the absence of drug.

The results from the experiment demonstrate variable gene expression between susceptible and resistant isolates. FLC susceptible isolates began overexpressing *CDR2*, *MDR1*, and *ERG11* at 4 hours of drug exposure. FLC exposure does not affect *CDR1* expression in any of the four isolates with the exception of isolates 1 and 4 at 32 hours. As the target of FLC, *ERG11* overexpression is induced by drug in all strains. Susceptible isolates begin overexpressing resistance genes earlier and at higher levels than resistant isolates that express high basal gene expression in the absence of drug. Therefore, gene overexpression of resistance genes in response to the exposure time of FLC at MIC_{80} concentrations varies depending on the resistant gene, and its basal expression levels in resistant isolates.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Biological Sciences, have examined a thesis titled "Expression Pattern of Drug-Resistance Genes in Matched Clinical Isolates of *Candida albicans* at Variable Fluconazole Exposure Times" presented by Eric Stephen Geanes, candidate for the Master of Science degree, and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

Theodore C. White, Ph.D., Committee Chair

Department of Cell Biology and Biophysics

Thomas M. Menees, Ph.D.

Department of Cell Biology and Biophysics

Marilyn D. Yoder, Ph.D.

Department of Cell Biology and Biophysics

CONTENTS

ILLUSTRATIONS

TABLES

ACKNOWLEDGEMENTS

This research was funded by unrestricted research funds from the School of Biological Sciences, University of Missouri-Kansas City (UMKC). I would like to thank Ted White, Aditi Khanna, Somanon Bhattacharya, Brooke Esquivel, Edwin and Leanne Geanes for their helpful contributions and support.

CHAPTER 1

INTRODUCTION

Candida albicans is an opportunistic pathogenic fungus that mainly affects immunocompromised patients, such as individuals with AIDS, organ transplant recipients, and cancer patients receiving chemotherapy (2). Candidiasis infections can become life threatening and are estimated to kill over 400,000 individuals each year worldwide (3). Most antifungal drugs used to treat infections target ergosterol, the major sterol in the fungal plasma membrane. Ergosterol is vital for membrane fluidity and contributes to many essential cellular functions in fungi. Ergosterol biosynthesis inhibitors, such as azoles, target enzymes that act on ergosterol precursors. Fluconazole (FLC), a common azole, inhibits lanosterol 14α-demethylase (Erg11), preventing conversion of lanosterol to 4,4-dimethyl-cholesta-8,14,24-trienol and disrupting ergosterol production (4).

Previous studies have reported that administration of low dose azole antifungals for extended periods of time result in development of azole resistance in *Candida albicans* (2). The mechanisms involved in increasing resistance to azoles in pathogenic fungi are an important area of research and several contributing factors have been identified. Drug efflux pumps, such as ATP-binding cassette transporters (ABC transporters) and major facilitator transporters (MFS transporters), have been shown to be associated with increase in FLC resistance (4, 5). The transporters, specifically the ABC transporters Cdr1, Cdr2, and MFS transporter Mdr1, reside in the membranes of the cell and actively pump FLC out of the cell. Resistance increases as high levels of pumps decrease the intracellular FLC concentrations. Another factor leading to resistance is overexpression of Erg11, the target of FLC. Resistance

increases as the amount of lanosterol 14α-demethylase increases, requiring increased concentrations of FLC to inhibit the production of ergosterol (2).

Seventeen clinical isolates of *C.albicans* were obtained from an individual AIDS patient being treated with FLC over a two year time period. The FLC concentration used for treatment was increased to control the infection. The minimum inhibitory concentrations (MIC_{80}) was determined for each isolate, with isolate 1 being the earliest sample and the most susceptible and isolate 17 being the latest sample and most resistant. mRNA overexpression of *CDR1, CDR2, MDR1*, and *ERG11* were observed to occur with each increase in resistance in the isolates. Overexpression of *MDR1* first occurs in isolate 2, *ERG11* overexpression first occurred in isolate 13, and *CDR1* and *CDR2* overexpression first occurs with isolate 16 (Figure 1) (2, 6). The overexpression of *MDR1, ERG11,* and *CDR1/CDR2* is caused by mutations in the transcription factors *MRR1, UPC2,* and *TAC1,* respectively (7-9).

Further analysis was conducted on four isolates from the matched series (1, 4, 13, and 17), each of which has a unique pattern of overexpressed resistance genes. mRNA expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11* was measured at varying FLC concentrations dependent on each isolate's MIC_{80}. Results indicate maximum overexpression of each gene occurred at or near each isolate's MIC_{80}. Isolate 1 had maximum overexpression for *MDR1*, *ERG11*, *CDR1*, and *CDR2*. Isolate 4 had maximum overexpression for *ERG11*, *CDR1*, and *CDR2*. Isolate 13 had maximum overexpression for *CDR1*, and *CDR2*. Isolate 17's MIC_{80} cannot be measured due to solubility limitations of FLC and therefore does not have a MIC_{80}. Isolate 17 did not have any maximum overexpression. Maximum gene expression occurred at

each isolate's MIC_{80}, except when a gene was overexpressed without drug, in which case no further overexpression was observed (1).

This paper investigates how mRNA expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11* vary in the four clinical isolates for six time points after FLC exposure of 1, 2, 4, 8, 16, and 32 hours. Drug concentrations were set at each isolates' MIC_{80} for maximum expression of each gene at all six time points.

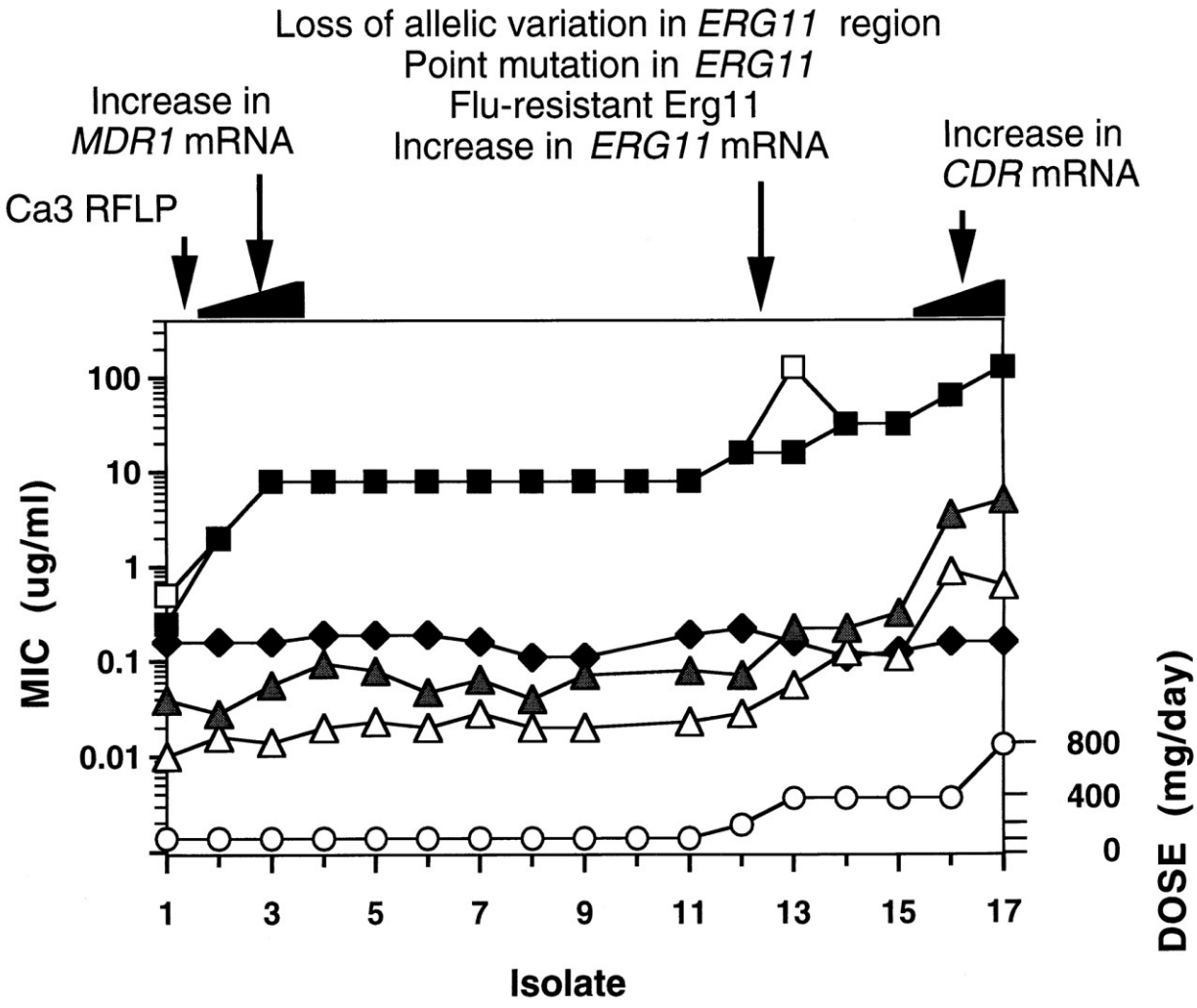


Figure 1: Seventeen Clinical Isolates from a Single Patient. Left Y axis shows MIC to FLC (filled and open boxes), itraconazole (filled triangles), ketoconazole (open triangles), and amphotericin B (filled diamonds). Right Y axis shows the dose of azoles given to patient. Top of graph indicates changes that occur at each arrow. *MDR1*, *ERG11*, and *CDRs* overexpression is indicated at Isolate 2, 13, and 16 respectively. (6)

CHAPTER 2

MATERIALS AND METHODS

Growth of Culture and Isolates

Four isolates of *Candida albicans* were chosen for this project from 17 clinical isolates taken from the same patient over the course of two years (Table 1)(10). Overnight cultures were prepared by inoculating CSM Complete (0.8 g complete supplement mixture, 1.7 g yeast nitrogen base without amino acids and ammonium sulfate, 5 g ammonium sulfate per liter) with 2% glucose and grown at 30° C. For analysis, samples were grown in liquid CSM complete media with 2% glucose.

Table 1: Clinical Isolates

Isolate Name	TW Catalog Number	Isolate Number
Isolate 1	TW 072-28	2-76
Isolate 4	TW 072-31	2-81
Isolate 13	TW 072-39	8-44
Isolate 17	TW 072-43	12-99

Minimum Inhibitory Concentration

Susceptibility to FLC was measured for the four clinical isolates using the CLSI-approved microbroth microdilution protocol (11). Susceptibility was expressed as the minimum inhibitory concentrations of FLC for the isolates, calculated as the amount of drug

that reduces cell growth by 80% (11). Cells were grown in CSM complete media with two-fold serial dilutions of drug in 96 well plates for 48 hours at 30º C. Cell growth in the presence of drug was normalized to the positive control wells exhibiting cell growth in the absence of drug. A row of wells in the 96 well plate contain media without cells to provide a negative growth control for each sample. All media and reagents were ordered from Sigma-Aldrich, Fisher Scientific, and Sunrise Science Products.

Quantitative Real Time PCR

Cultures were inoculated in 500 ml CSM complete at an A_{600} measurement of 0.1 from a single colony for each isolate. For each of the four isolates, cells were grown in media without drug, and media with drug at concentrations corresponding to the MIC_{80} of the isolate. RNA was extracted using Qiagen RNeasy mini purification kit at 1, 2, 4, 8, 16, and 32 hours post-inoculation. RNA concentrations and purity were measured using the A_{260}/A_{280} ratio in a multimode plate reader (Biotek, Winooski, VT). RNA extracts were electrophoresed in 1.2% agarose gels to identify separate ribosomal RNA bands to verify that there was minimal RNA degradation for each sample. Superscript III First-Strand kit (Invitrogen, Waltham, MA) was used to produce cDNA from mRNA concentrations equalized to identical concentrations of 525 ng with RNAse free water. Quantitative Real Time PCR (7500 Real-Time PCR system, Applied Biosystems) was performed using the manufacturers protocol (Thermo Scientific Maxima SYBR Green RT-PCR kit). Primers previously used to evaluate the mRNA expression levels of drug resistance genes in *C. albicans* were used to amplify the genes, listed in Table 2 (12).

Table 2: qRT-PCR Primers			
Gene	**Forward Primer**	**Reverse Primer**	
ACT1	GAAGAAGTTGCTGCTTTAG	CGTCGTCACCGGCAAAA	(13)
CDR1	AAGATGTCGTCGCAAGATGAAT	GAGTGAAAGTTCTGGCTAAATTC	(13)
CDR2	TTGAGCCACATGTCCGACAT	GGAATCTGGGTCTAATTGTTCAT	(13)
MDR1	ATCACCGGTAACGACAGAATCA	TCTAATGGTCTCCATAATGTATCAATGA	(13)
ERG11	CCCCTATTAATTTTGTTTTCCCTAATTTAC	CACGTTCTCTTCTCAGTTTAATTTCTTTC	(13)

For each time point, the expression data of mRNA present in each cell for each gene was normalized to β-actin gene expression. For each time point, the relative expression levels of resistance genes in the presence of drug were compared to the relative expression levels in the absence of drug. The specificity of primers was determined with a dissociation step immediately after each experiment. All qRT-PCR experiments were performed in biological triplicate. Expression data calculated fold changes as $2^{-\Delta\Delta Ct}$ with significant values being two fold greater or less than the no drug controls. Statistical analysis was done using unpaired t-test using GraphPad Prism 6. Error bar calculation was consistent with previously described methods (12). Gene expression differentials are significant when above or below 2 fold the mRNA expression corresponding to the relative no drug controls.

CHAPTER 3

RESULTS

Minimum Inhibitory Concentrations (MIC_{80})

As an initial confirmation of the clinical isolates have consistent FLC resistance with previous studies, the MIC_{80} to FLC was determined for each of the four clinical isolates to be used in this study (Table 1). Resistant strains are considered to have an MIC_{80} of ≥8 μg/ml (14-17). Resistance to FLC increased with each isolate. Isolate 1 was the most susceptible of the four with 80% reduction in growth at 1 μg/ml, Isolate 4 was more resistant than isolate 1 at 8 μg/ml, Isolate 13 was more resistant than isolate 4 at 32 μg/ml, and isolate 17 was most resistant of all four isolates with a MIC value of ≥128 μg/ml (Figure 2). These results are consistent with previous findings (6).

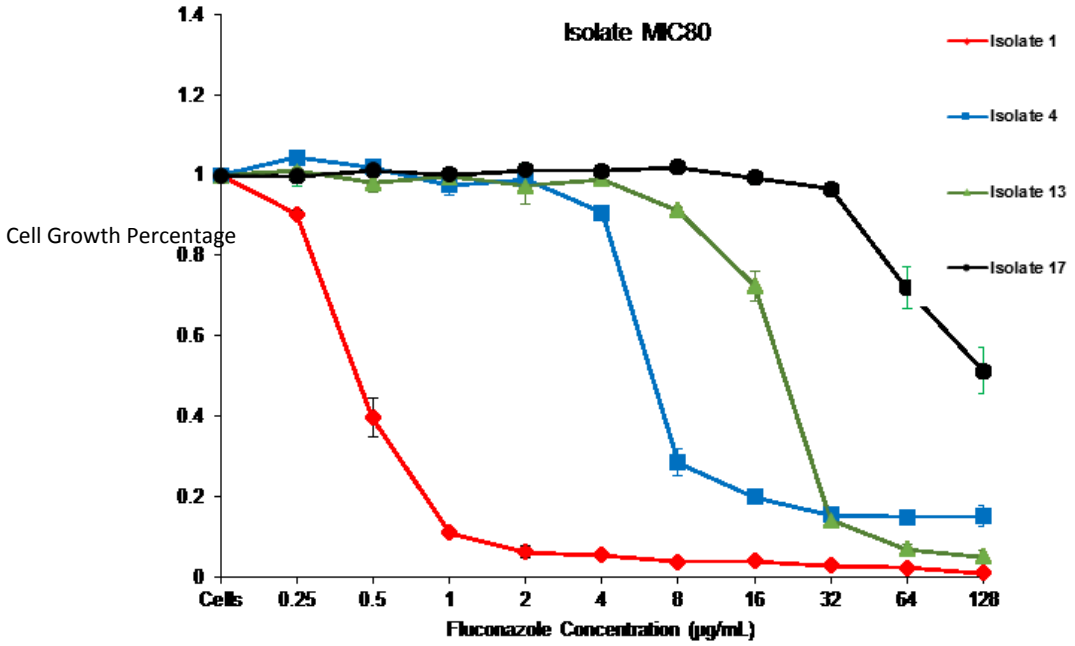


Figure 2: MIC_{80} of Isolates 1, 4, 13, 17: 48 hour growth of isolates in CSM complete liquid media. Minimum inhibitory concentrations can be calculated at the drug concentration with 80% growth inhibited. Graph represents percent cell growth in relation to a no drug control over increasing FLC concentration. Isolate 1 is represented as red diamonds, isolate 4 as blue squares, isolate 13 as green triangles, and isolate 17 as black circles.

Quantitative Real Time PCR Analysis: Expression of mRNA encoding for efflux pumps and lanosterol 14α-demethylase genes

To determine the response of the four matched clinical isolates, the gene expression pattern was determined at the MIC_{80}, as previously described. This analysis focused on the timing of gene overexpression at the FLC MIC_{80} concentration of each isolate, using times 1, 2, 4, 8, 16 and 32 hours. A control in which cells were grown in the absence of drug was also prepared and used for normalization.

Table 3: Isolate gene expression without FLC exposure
Bold boxes were found to be statistically significant.

	Time (h)						
Genes	**1**	**2**	**4**	**8**	**16**	**32**	**Isolates**
CDR1	1.00	1.00	1.00	1.00	1.00	1.00	**1**
	0.92	0.65	0.76	1.00	5.72	0.94	**4**
	0.57	0.34	0.52	0.72	1.24	0.89	**13**
	12.72	4.38	5.34	4.38	26.75	4.99	**17**
CDR2	1.00	1.00	1.00	1.00	1.00	1.00	**1**
	2.99	1.48	2.01	1.27	1.07	3.03	**4**
	6.22	1.83	1.74	6.68	2.34	5.38	**13**
	918.59	268.13	136.49	148.45	703.88	731.57	**17**
MDR1	1.00	1.00	1.00	1.00	1.00	1.00	**1**
	34.26	25.96	7.50	28.89	23.70	17.64	**4**
	40.85	24.06	7.46	15.07	29.25	146.26	**13**
	47.92	55.07	10.76	36.30	28.91	101.61	**17**
ERG11	1.00	1.00	1.00	1.00	1.00	1.00	**1**
	0.78	0.83	0.78	1.11	2.65	14.49	**4**
	1.17	1.42	0.84	4.14	0.89	2.79	**13**
	2.05	2.56	1.20	3.15	5.85	4.90	**17**

Table 3: qRT-PCR expression levels in the absence of FLC normalized to isolate 1 for each time point. Each gene is color coded for the lowest values shaded blue and the highest values shaded orange.

The mRNA expression difference between matched isolates in the absences of FLC was evaluated by comparing the three resistant isolates (4, 13, 17) to susceptible isolate 1 at each time point (Table 3). Isolate 1 exhibits the lowest expression of *MDR1* and *CDR2* at all time points compared to the other isolates (blue in Table 3). Similarly, isolate 1 exhibits low expression of *ERG11* and *CDR1* at most time points compared to the other isolates. All

isolates reached their lowest expression of all four genes at or near 4 hours of growth (columns in Table 3), but expression increased to higher levels at eight to 16 hours of growth for isolates with gene resistance mutations.

For *CDR1*, isolates 4 and 13 had expression levels below isolate 1, while isolate 17 had overexpression for *CDR1*, especially at one and 16 hours. For *CDR2*, isolate 17 exhibited very high levels of expression at all time points, isolate 13 exhibited elevated levels of expression, and isolate 4 exhibited no significant change compared to isolate 1. For *MDR1*, all three resistant isolates exhibited overexpression for all time points. For *ERG11,* isolate 4 showed low expression from one to four hours, but increased to overexpressed levels by 32 hours. Both isolates 13 and 17 overexpressed *ERG11* after eight to 32 hours. These results are consistent with previous work describing the genes overexpressed by the resistant isolates with no exposure to FLC (2, 6). However, previous work has not described gene expression over time for these or other clinical isolates.

The mRNA expression of resistance genes from matched isolates in the presence of FLC at the MIC_{80} concentration was compared to mRNA expression of the isolates grown in the absence of drug at each time point (Table 4). For *CDR1*, exposure of all four isolates to FLC at MIC_{80} concentrations had little effect on gene expression, except perhaps at 32 hours. At 32 hours, isolates 1 and 13 had only minor non-significant increases in expression. Isolate 4 significantly under expressed *CDR1* at 4 hours, while over-expressing the gene after 32 hours in FLC. Isolate 17 under expressed *CDR1* at 16 hours, but FLC had no affect at any other length of exposure.

Table 4: qRT-PCR Summary of gene expression after varied time exposed to MIC_{80} FLC concentrations. Bold boxes were found to be statistically significant. *Isolate 17 does not have a MIC80 concentration of FLC.

	Time (h)								
Genes	**No Drug**	**1**	**2**	**4**	**8**	**16**	**32**	**Isolates**	**FLC Conc (µg/ml)**
CDR1	1	1.60	1.38	0.88	1.01	0.70	1.92	**1**	1
	1	0.79	2.67	0.66	1.77	0.52	1.77	**4**	8
	1	0.85	1.94	1.14	1.31	0.99	1.93	**13**	32
	1	0.93	1.58	0.85	2.18	0.21	1.66	**17**	128*
CDR2	1	1.19	4.88	4.72	3.64	1.14	17.82	**1**	1
	1	0.72	1.16	1.80	37.59	3.59	3.03	**4**	8
	1	0.80	1.33	1.36	2.04	4.33	1.81	**13**	32
	1	1.02	0.69	0.83	2.07	0.31	1.06	**17**	128*
MDR1	1	1.12	1.25	4.68	4.65	0.46	38.11	**1**	1
	1	1.12	1.79	0.95	0.93	4.02	11.93	**4**	8
	1	0.92	2.02	0.96	2.00	2.10	1.10	**13**	32
	1	0.69	0.84	0.46	2.40	1.36	0.85	**17**	128*
ERG11	1	1.26	1.43	2.21	16.13	6.77	10.19	**1**	1
	1	1.52	1.50	1.69	2.42	1.84	7.22	**4**	8
	1	0.95	1.09	2.26	2.31	9.83	7.61	**13**	32
	1	0.79	0.98	2.09	3.87	1.66	5.13	**17**	128*

Table 4: qRT-PCR expression levels in the presence of FLC normalized to no drug control for each isolate at each FLC exposure time. Each gene is color coded for the lowest values shaded blue and the highest values shaded orange. Dark outlined boxes are statistically significant.

For *CDR2*, isolates 1, 4, and 13 all began increasing expression of *CDR2* after 4 to 8 hours of FLC exposure and continued to overexpress the gene up to 32 hours, but these values did not become significant. Maximum expression in isolate 1 was after 32 hours, while maximum expression in isolate 4 was eight hours and in isolate 13 was 16 hours. In Isolate 17, expression of *CDR2* was significantly under expressed after both two hours and 16 hours of FLC exposure, but there was no affect at other exposure times. For *MDR1*,

isolates 1 and 4 began increasing expression of *MDR1* at four and 16 hours respectively, but did not significantly over express until isolate 4 grew for 32 hours of FLC exposure. Isolates 13 and 17 had no significant change in expression of *MDR1*. *ERG11* began to be significantly overexpress after four hours of FLC exposure for all four isolates and continued overexpression at subsequent time points.

CHAPTER 4

DISCUSSION

The goal of this study is determine the variation in mRNA expression associated with FLC resistance in *Candida albicans* in relation to FLC exposure over time at clinical isolate MIC_{80} concentrations. Isolates 4, 13, and 17 overexpress genes in accordance with increasing resistance to FLC compared to isolate 1 (6). However, mRNA expression in relation to increasing FLC concentration or increasing time of FLC exposure was not known. A previous investigation from this laboratory of FLC concentration in relation to resistant gene mRNA expression revealed maximum gene expression occurred at or near the MIC_{80} concentration of each clinical isolate (1). In this study, mRNA expression of these genes was measured after specific time intervals exposing the isolates to their MIC_{80} concentration of FLC. Gene expression was measured for each isolate in the both the presence and absence of FLC.

Comparison between basal gene expression levels without FLC exposure at each time point confirms the susceptibility of isolate 1 with no increased expression for *MDR1*, *ERG11*, *CDR1*, or *CDR2* (Table 3). Isolate 4 was consistent with previous findings of resistance coinciding with elevated gene expression for *MDR1* (6). Isolate 13 increases resistance while overexpressing both *MDR1* and *ERG11*, whereas isolate 17 maintains the highest resistance in the series of the isolates while also overexpressing *MDR1*, *ERG11*, *CDR1*, and *CDR2.*

The mRNA expression in the isolates exposed to FLC at respective MIC_{80} concentrations were normalized to controls grown in the absence of FLC for the same time

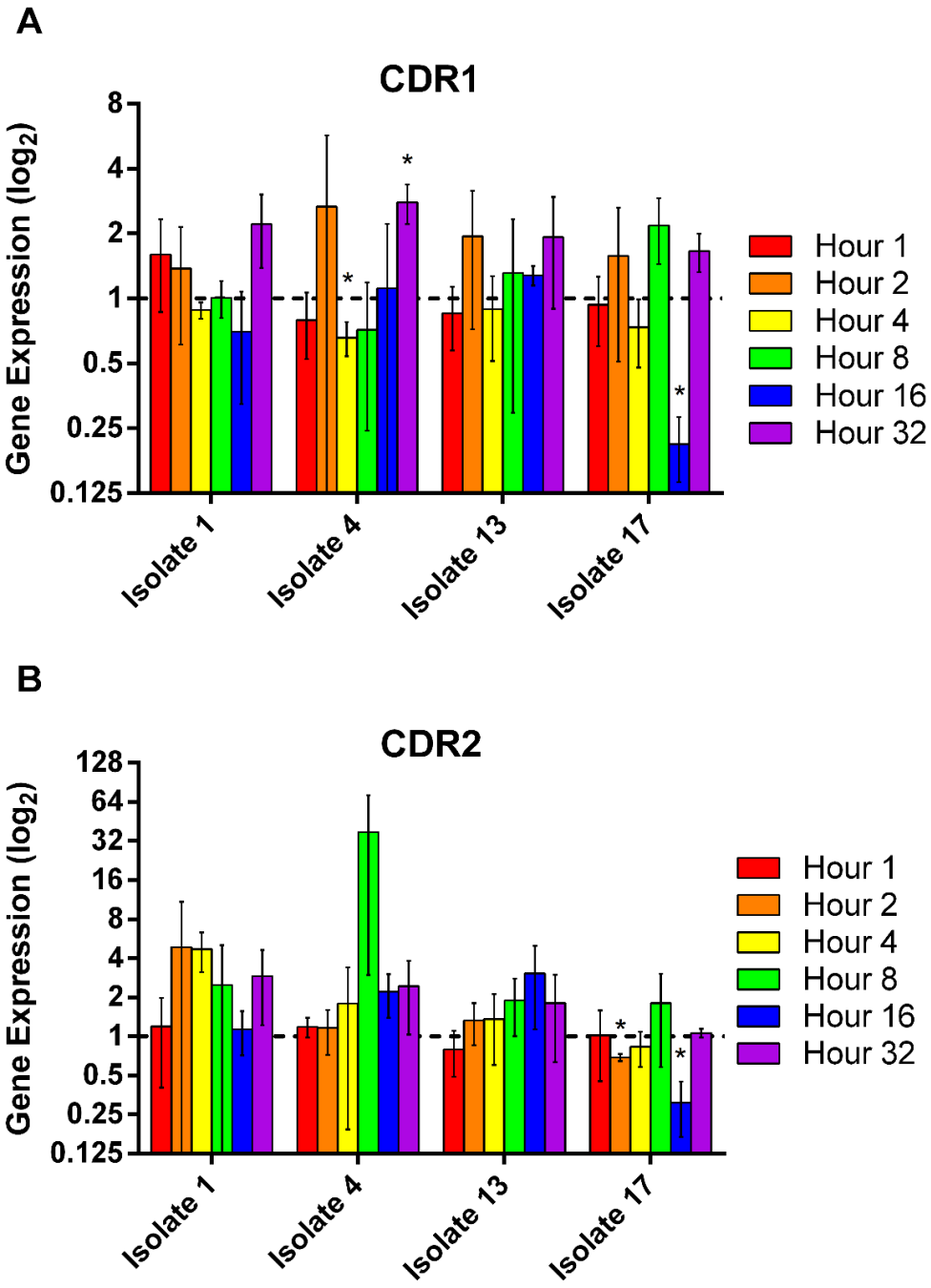
period. *CDR1* expression was under expressed or was not effected in all four isolates with the exception of 32 hours of FLC exposure. At 32 hours, isolates 1 and 4 began over expressing *CDR1*. Isolate 13 only had minor increases in expression, while isolate 17 remained unaffected (Figure 3A). Expression of *CDR1* in these isolates is not affected by the presence of FLC. *CDR2* expression began to increase after 2 hours of FLC exposure in isolates 1, 4, and 13, whereas Isolate 17 under expressed after 2 hours (Figure 3B). Isolates 1, 4, and 13 do not overexpress *CDR2* in the absence of drug (8), so the presence of drug may still induce the stress on the cell and increase pump expression. Isolate 17 overexpresses all four resistance genes at basal levels and therefore does not require increased expression to maintain growth in the presence of FLC (6).

MDR1 had increased expression in Isolates 1 and 4 after four to eight hours of exposure to FLC. Isolate 13 had no significant change in *MDR1* expression over time, while Isolate 17 had no change but under expressed *MDR1* at hour two (Figure 3C). Isolates 1 and 4 are susceptible or maintain only one source of resistance to FLC respectively. Without basal overexpression or additional resistance genes to counter the effects of FLC, increased expression of *MDR1* is necessary for cell growth to proceed to the exponential phase. Isolates 13 and 17 basal overexpression of *MDR1* and additional sources of resistance from *ERG11* (13 and 17), *CDR1,* and *CDR2* (Isolate 17) account for the lack of change in expression over time.

ERG11 overexpression began around four hours for all isolates and continued to increase through 32 hours (Figure 3D). This continuous increase in expression from four hours to 32 hours could be due to Erg11 being the main target of FLC. Isolates must

compensate for elevated FLC by producing more Erg11 to maintain healthy ergosterol levels in cells for proper growth throughout and past the exponential phase.

The response to FLC at MIC_{80} concentrations results in increases in expression of resistant genes in isolates 1, 4, and 13. Isolate 17 was not grown at MIC_{80} concentrations and maintains resistance through higher basal expression levels of *CDR1, CDR2, MDR1,* and *ERG11.* There is a decrease in genes being overexpressed in resistant strains as resistance increases, shown as the difference between isolate mRNA expression of *MDR1* and *CDR2*. The ability or necessity for isolate 17 to use valuable resources to increase expression may not be present without the negative effects on growth at the MIC_{80} concentration the other isolates encounter.


A
CDR1
Gene Expression (log2)
8
4
2
1
0.5
0.25
0.125
Isolate 1
Isolate 4
Isolate 13
Isolate 17
Hour 1
Hour 2
Hour 4
Hour 8
Hour 16
Hour 32
B
CDR2
Gene Expression (log2)
128
64
32
16
8
4
2
1
0.5
0.25
0.125
Isolate 1
Isolate 4
Isolate 13
Isolate 17
Hour 1
Hour 2
Hour 4
Hour 8
Hour 16
Hour 32

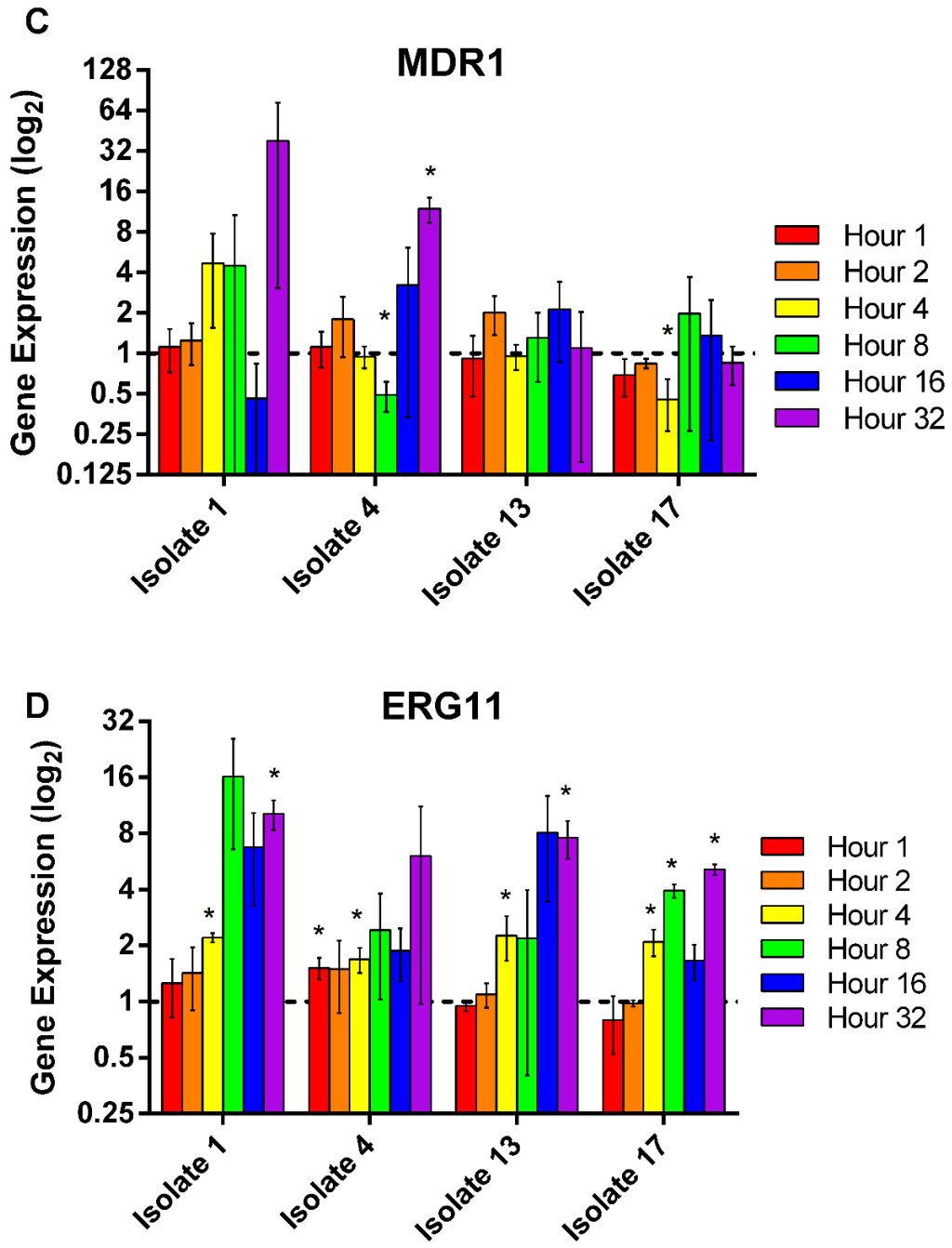


Figure 3: mRNA Expression levels: Matched isolates expression of the genes a) *CDR1,* b) *CDR2,* c) *MDR1,* and d) *ERG11* at time points in biological triplicates. All data was normalized to cells grown in the absence of FLC for each isolate at each time point and *ACT1* as a control. Hour 1 is shown in red, hour 2 in orange, hour 4 in yellow, hour 8 in dark green, hour 16 in blue, and hour 32 in purple.

ERG11 continues to be over expressed in all isolates which has a different trend than shown with *CDR1* and *MDR1*. This overexpression for all four isolates may be caused by an unknown source since the presence of the *ERG11* transcription factor mutation in Upc2 does not seem to make a difference in overexpression. Alternatively, the resistance could be due to FLC targeting lanosterol 14α-demethylase (Erg11) (2). Cellular response to FLC targeting Erg11 results in a negative feedback system. FLC inhibits Erg11, resulting in low levels of ergosterol in the cell. These low levels trigger an increase in the transcription factor *UPC2* to initiate increased *ERG11* expression and in turn increased Erg11 in the cell to increase ergosterol in the cell (18). A previous study reveals isolates 1 and 4 without *ERG11* mutation continuously increase expression as FLC concentration increases. With the development of the *ERG11* mutation in isolate 13, overexpression only begins with a rapid increase in expression at the MIC_{80} and higher concentrations(1). While susceptible isolates need to continuously increase Erg11 to combat the effects of FLC, resistant isolates with the *ERG11* mutation only see increases in expression once FLC concentrations reach their MIC_{80}. After this concentration is reached, the isolates no longer produces an adequate amount of Erg11 to counter the fungistatic effect of FLC, and must begin overexpressing *ERG11*. As the isolates are grown in the presence of FLC at their MIC_{80} concentrations, inhibition of Erg11 from the start of growth and continuing for all time points increases the necessity of expressing *ERG11*. The cells may attempt to neutralize the effect of FLC, which would result in proper ergosterol synthesis and lipid fluidity within the cell. Even at 32 hours of growth, the MIC_{80} concentration requires such a high Erg11 threshold to neutralize FLC effects that cells continue to increase *ERG11* expression.

Consolidating average mRNA expression results between this study and the previous work done in the lab that investigated mRNA expression at FLC concentrations provides an increased understanding of the relationship between concentration and time of exposure of FLC for these matched isolates of *C. albicans*. Maximum expression for each gene is dependent on FLC concentration, having the highest value at the MIC_{80} concentration. Gene expression levels in response to time of FLC exposure is dependent on basal gene expression, which is influenced by transcription factor mutations that result in overexpression even in the absence of drug, and constant drug targeted stress on vital enzymes.

CDR1 and *CDR2* expression is maximized near the MIC_{80} concentration, but only isolates 1, 4, and 13 (see increases in gene expression at MIC_{80} concentration; Figures 4A, 4B). The isolates do not contain the mutation in the transcription factor *TAC1*, which is specific to isolate 17 and causes higher basal *CDR1* and *CDR2* expression (8). Without the *TAC1* mutation allowing isolate 17 to have constant elevated *CDR1* and *CDR2* levels, isolates 1, 4, and 13 increase expression of the ABC transporters to combat the presence of FLC.

MDR1 expression is maximized near the MIC_{80} concentration, but only isolates 1 and 4 (see increases in gene expression at MIC_{80} concentration; Figures 4C). Isolate 1 does not contain a mutation in the transcription factor *MRR1* that is specific for increased resistance in isolates 4, 13, and 17 (9). Although isolate 4 does start to overexpress *MDR1* at 16 hours of FLC exposure, response time is delayed compared to susceptible isolate 1 overexpression occurring after only 4 hours. Isolate 4 is only slightly resistant as it does not have the additional resistance genes overexpressed that occur in isolates 13 and 17. With only one

source of resistance reported, producing more major facilitator transporters may be essential for isolate 4 to survive longer FLC exposure.

ERG11 expression is maximized near the MIC_{80} concentration for isolates 1, 4, and 13, and continuously increases expression throughout all time points for all four isolates (Figures 4D). Isolate 17 has an MIC_{80} for FLC higher than achievable concentrations. Therefore the MIC_{80} FLC concentration that would trigger *ERG11* overexpression cannot be created *in vitro*. The overexpression of *ERG11* in response to both concentration and time exposed to FLC is the result of FLC targeting the product of *ERG11*, lanosterol 14α-demethylase. Inhibiting Erg11 prevents conversion of lanosterol to 4,4-dimethyl-cholesta-8,14,24-trienol and disrupts vital ergosterol production. Isolates overexpress *ERG11* to counterbalance the effects of FLC, and thus continue to produce ergosterol and thrive. This overexpression over time appears to be independent of the known UPC2 mutation that occurs in Isolates 13 and 17 (19).

Identifying expression patterns of transcription factors contributing to resistance gene expression in response to FLC concentration and exposure time will help consolidate a stronger understanding for cellular response to drug treatments. Measuring mRNA expression does not confirm protein production from the overexpression of the genes. Monitoring efflux pump activity and protein expression through immunoblotting would support our finding that FLC at MIC_{80} concentrations induces higher gene expression and in turn induces higher protein production from the overexpression of resistant genes in susceptible isolates, but does not alter the concentrations of resistant isolates for resistant genes being constitutively overexpressed. The results of this study may contribute to future drug therapies contrived from drug dosage and time of release. Knowing which mechanisms

are initiated and the amount of time needed for resistant genes to overexpress after drug exposure will allow for multidrug treatments with a higher level of effectiveness of combating *Candida albicans*' ability to build resistance.

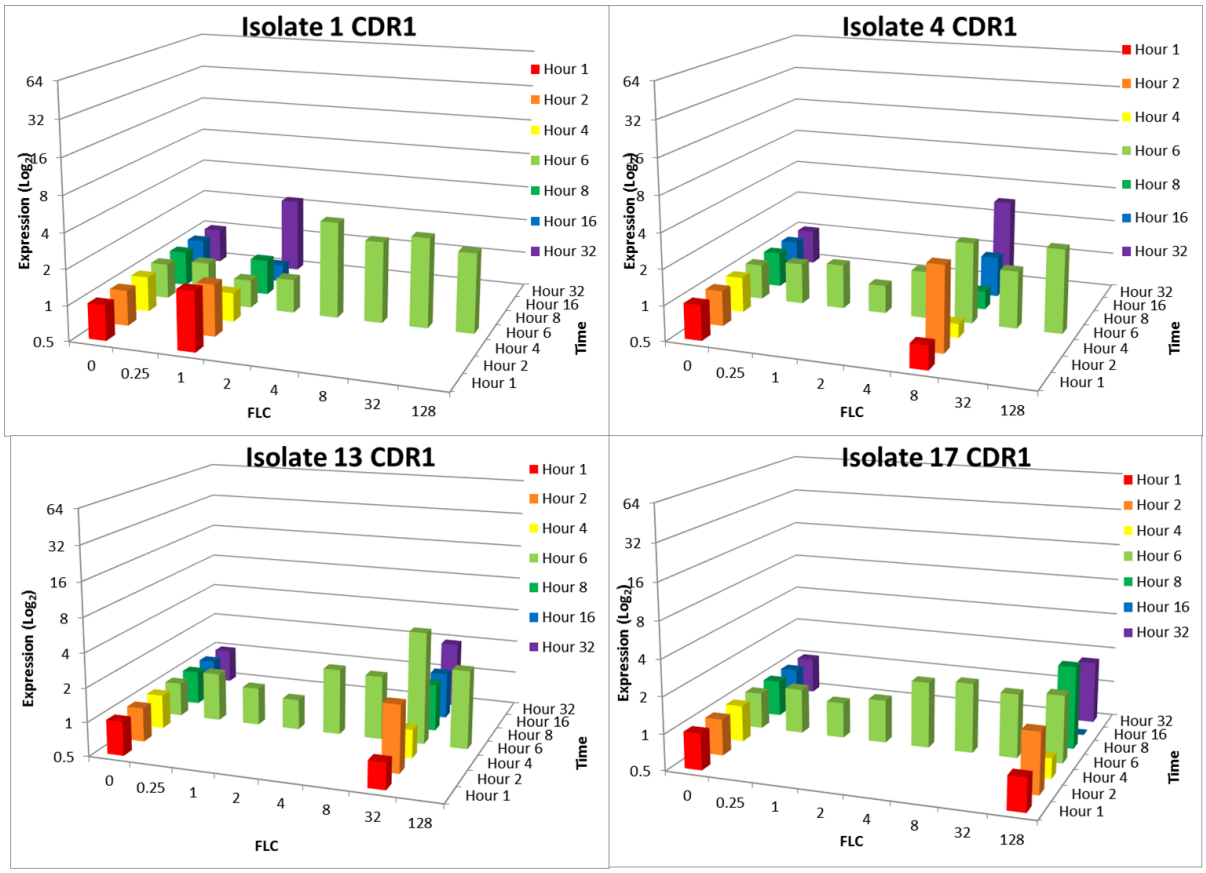


Figure 4A: FLC Concentration VS Exposure Time 3D Graphs of Average *CDR1* mRNA expression. Values are normalized to corresponding isolate in the absence of FLC for each time point. Hour 1 is shown in red, hour 2 in orange, hour 4 in yellow, hour 6 in light green, hour 8 in dark green, hour 16 in blue, and hour 32 in purple.

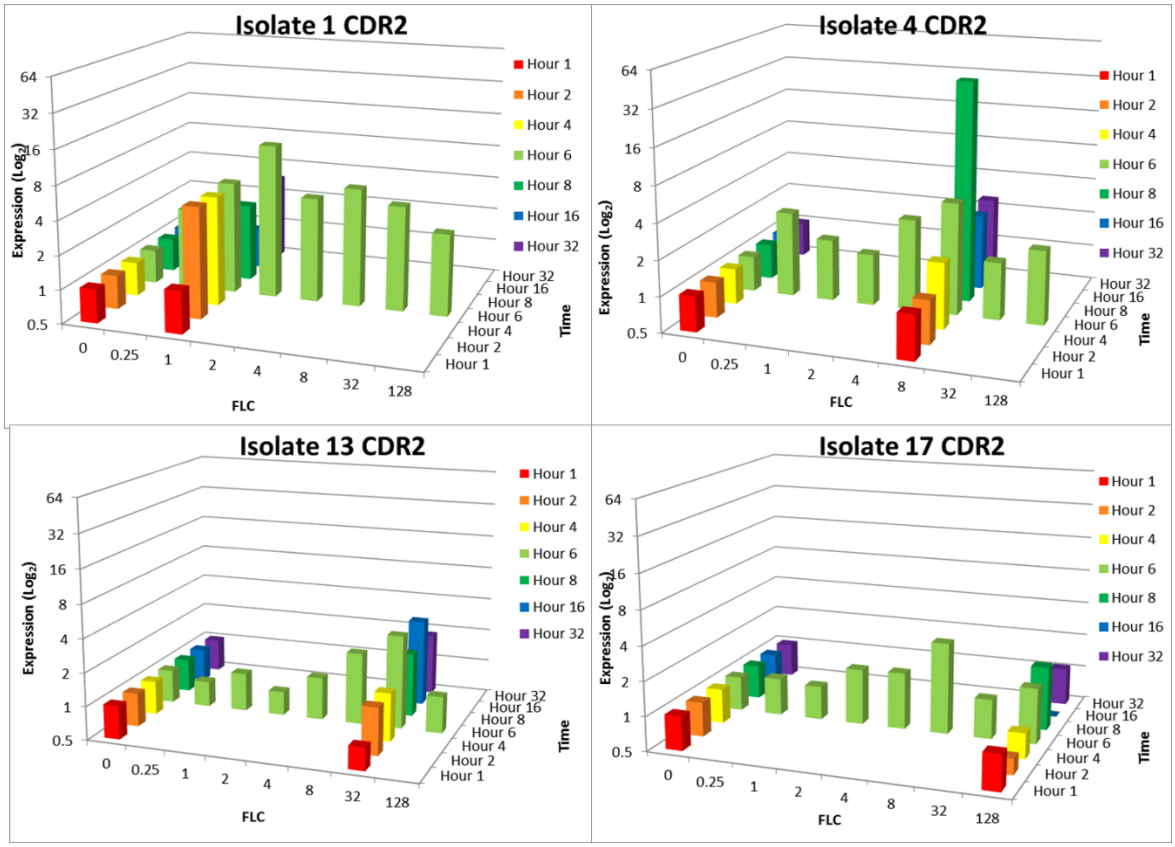


Figure 4B: FLC Concentration VS Exposure Time 3D Graphs of Average *CDR2* mRNA expression. Values are normalized to corresponding isolate in the absence of FLC for each time point. Hour 1 is shown in red, hour 2 in orange, hour 4 in yellow, hour 6 in light green, hour 8 in dark green, hour 16 in blue, and hour 32 in purple.

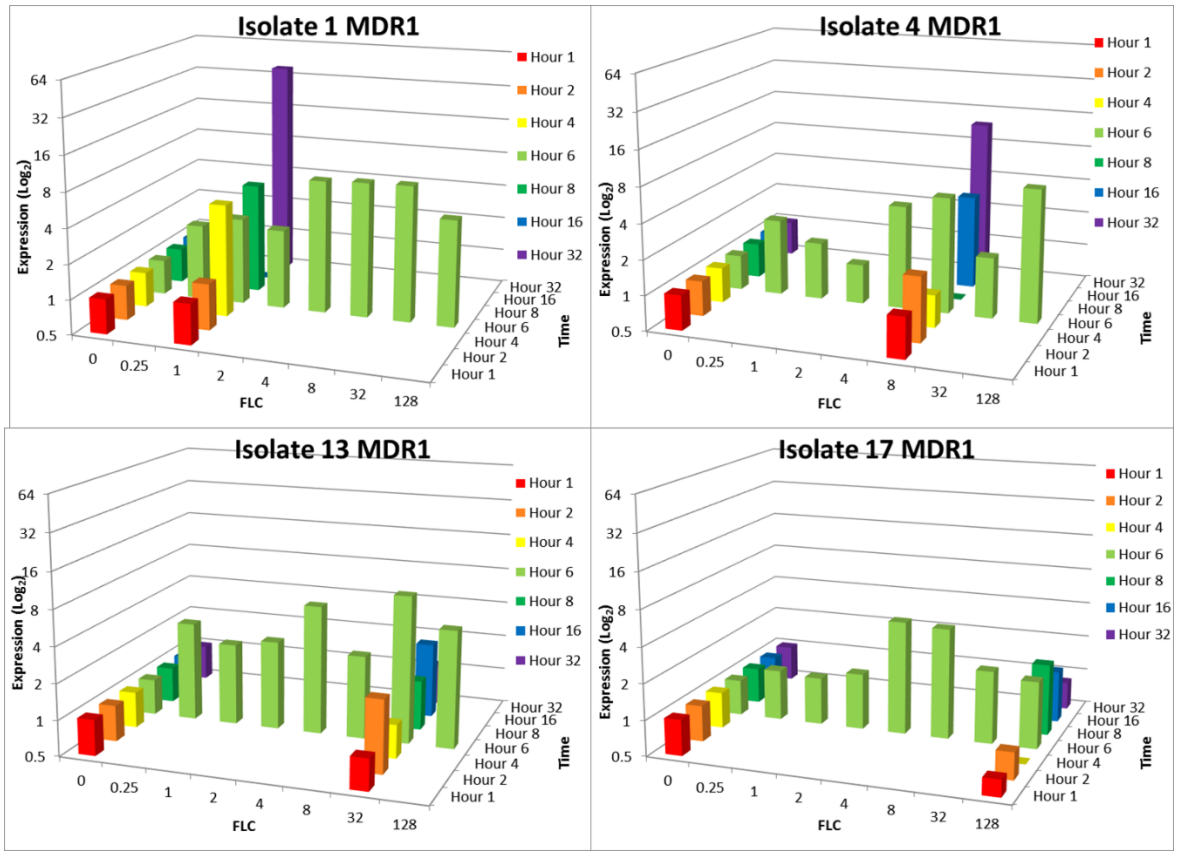


Figure 4C: FLC Concentration VS Exposure Time 3D Graphs of Average *MDR1* mRNA expression. Values are normalized to corresponding isolate in the absence of FLC for each time point. Hour 1 is shown in red, hour 2 in orange, hour 4 in yellow, hour 6 in light green, hour 8 in dark green, hour 16 in blue, and hour 32 in purple.

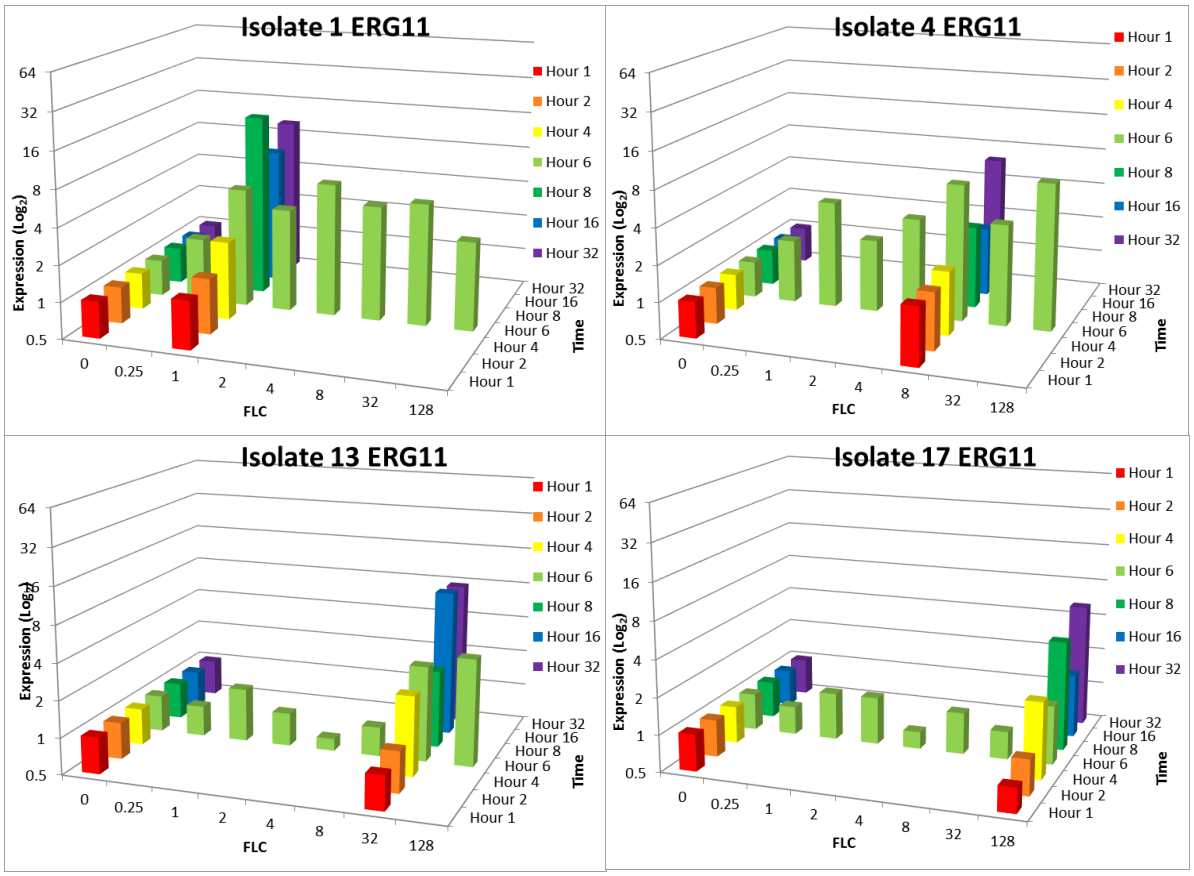


Figure 4D: FLC Concentration VS Exposure Time 3D Graphs of Average *ERG11* mRNA expression. Values are normalized to corresponding isolate in the absence of FLC for each time point. Hour 1 is shown in red, hour 2 in orange, hour 4 in yellow, hour 6 in light green, hour 8 in dark green, hour 16 in blue, and hour 32 in purple.

REFERENCES

1. **Khanna A.** 2015. Expression pattern of drug-resistance genes in *Candida albicans* at different Fluconazole concentrations. University of Missouri - Kansas City, School of Biological Sciences
2. **White TC, Marr KA, Bowden RA.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev **11:**382-402.
3. **Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC.** 2012. Hidden killers: human fungal infections. Sci Transl Med **4:**165rv113.
4. **Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J.** 2003. Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother **47:**2404-2412.
5. **Holmes AR, Keniya MV, Ivnitski-Steele I, Monk BC, Lamping E, Sklar LA, Cannon RD.** 2012. The monoamine oxidase A inhibitor clorgyline is a broad-spectrum inhibitor of fungal ABC and MFS transporter efflux pump activities which reverses the azole resistance of Candida albicans and Candida glabrata clinical isolates. Antimicrob Agents Chemother **56:**1508-1515.
6. **White TC.** 1997. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother **41:**1482-1487.
7. **Oliver BG, Song JL, Choiniere JH, White TC.** 2007. cis-Acting elements within the Candida albicans ERG11 promoter mediate the azole response through transcription factor Upc2p. Eukaryot Cell **6:**2231-2239.
8. **Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A, Berman J, Bille J, Sanglard D.** 2006. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics **172:**2139-2156.
9. **Dunkel N, Blass J, Rogers PD, Morschhauser J.** 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. Mol Microbiol **69:**827-840.
10. **White TC, Pfaller MA, Rinaldi MG, Smith J, Redding SW.** 1997. Stable azole drug resistance associated with a substrain of Candida albicans from an HIV-infected patient. Oral Dis **3 Suppl 1:**S102-109.
11. **Bertout S, Dunyach C, Drakulovski P, Reynes J, Mallie M.** 2011. Comparison of the Sensititre YeastOne(R) dilution method with the Clinical Laboratory Standards Institute (CLSI) M27-A3 microbroth dilution reference method for determining MIC of eight antifungal agents on 102 yeast strains. Pathol Biol (Paris) **59:**48-51.
12. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-408.

13. **Oliver BG, Silver PM, Marie C, Hoot SJ, Leyde SE, White TC.** 2008. Tetracycline alters drug susceptibility in Candida albicans and other pathogenic fungi. Microbiology **154:**960-970.
14. **Danby CS, Boikov D, Rautemaa-Richardson R, Sobel JD.** 2012. Effect of pH on in vitro susceptibility of Candida glabrata and Candida albicans to 11 antifungal agents and implications for clinical use. Antimicrob Agents Chemother **56:**1403-1406.
15. **Sobel JD, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H.** 2003. Fluconazole susceptibility of vaginal isolates obtained from women with complicated Candida vaginitis: clinical implications. Antimicrob Agents Chemother **47:**34-38.
16. **Suarez VL, Fernandez Andreu CM.** 2003. [In vitro susceptibility of vaginal isolates of Candida vs clotrimazole and nystatin]. Rev Cubana Med Trop **55:**138-145.
17. **Hamza OJ, Matee MI, Moshi MJ, Simon EN, Mugusi F, Mikx FH, Helderman WH, Rijs AJ, van der Ven AJ, Verweij PE.** 2008. Species distribution and in vitro antifungal susceptibility of oral yeast isolates from Tanzanian HIV-infected patients with primary and recurrent oropharyngeal candidiasis. BMC Microbiol **8:**135.
18. **Vasicek EM, Berkow EL, Flowers SA, Barker KS, Rogers PD.** 2014. UPC2 is universally essential for azole antifungal resistance in Candida albicans. Eukaryot Cell **13:**933-946.
19. **Hoot SJ, Brown RP, Oliver BG, White TC.** 2010. The UPC2 promoter in Candida albicans contains two cis-acting elements that bind directly to Upc2p, resulting in transcriptional autoregulation. Eukaryot Cell **9:**1354-1362.

VITA

Eric Stephen Geanes was born on January 24th, 1992 in Shawnee, Kansas. He was educated in the Blue Valley School District and graduated from Blue Valley West High School in 2010 after competing nationally in Science Bowl in Washington D.C. and receiving the Most Distinguished Senior in Sciences award.

In the fall of 2010, Eric received the Fairchild Scholarship and attended Kansas State University in Manhattan, Kansas. From his work studying the proteasome in Dr. Jeroen Roelofs' laboratory, he received the Michael F. Lukert Cancer Research Award in 2013, the James & Susan H. Ryan Cancer Research Award in 2014, and was a contributing author for the publication titled "1.15 Å Resolution Structure of the Proteasome Assembly Chaperone Nas2 PDZ Domain" in the journal Acta Crystallographica Section F. He graduated from Kansas State University in May of 2014 with the degree of Bachelor of Science in Biology.

In August of 2014, he began the master's program at the University of Missouri-Kansas City studying cellular and molecular biology. After a short research rotation in fall of 2014, Eric joined Dr. Theodore White's laboratory in May of 2015 to study drug resistance in pathogenic fungi. Upon completion of his degree, he plans to continue his scientific endeavors in industry or at a national laboratory.

Eric participates annually in the Kansas Mission of Mercy, Missouri Mission of Mercy, and is an active and founding member of the Kansas City Professional Chapter of Alpha Chi Sigma, Professional Chemistry Fraternity.